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14. ABSTRACT

The overall purpose of this research is to develop a polymeric drug delivery system containing magnetic resonance contrast agents for the treatment of breast cancer. This drug-imaging agent delivery system will allow the follow up of the fate of the drug delivery system and its relation to reduced tumor mass, improved efficacy and reduced toxicity in individual patients. In year two progress was made in the following areas: 1) Synthesis of polymer- contrast agent- doxorubicin conjugates; 2) Physicochemical characterization of polymer- contrast agent- doxorubicin conjugates; and 3) Relaxivity measurements. In addition, a series of polymer- contrast agent conjugates targetable to macrophages were synthesized, characterized, and evaluated in vitro.

15. SUBJECT TERMS
Polymers, drug delivery, breast cancer, contrast agent, MRI.

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INRODUCTION

The **long term objective** of this research is to develop a polymeric drug delivery system containing magnetic resonance contrast agents for treatment of breast cancer. This drug-imaging agent delivery system will allow the follow up of the fate of the drug delivery system and its relation to reduced tumor mass, improved efficacy and reduced toxicity in individual patients. Two specific aims were proposed:

1) To synthesize a series of polymer-drug-imaging agent conjugates.

2) To characterize the conjugates by physicochemical methods.

In year two of this project, progress was made to partially accomplish Aims 1 & 2 using gadolinium as a contrast agent. In doing so Tasks 2 (synthesis), 3 (characterization), and 4 (concluding year 2 / strategizing for year 3) of year two and Task 1 of year three (relaxivity measurements) outlined in the Statement of Work were accomplished. In addition related areas for targeted delivery to macrophages were explored as outlined in the body of this report.

BODY

A. Synthesis of the proposed comonomers and copolymers

We made progress in synthesis and characterization of the copolymers (Appendix 1, Fig 1). N-(2-hydroxypropyl)methacrylamide (HPMA)[1], and Methacryloylglycylphenylalanylleucylglycyl-doxorubicin (MA-GFLG-dox), a reactive comonomer with a lysosomally degradable linker[2] were prepared as described previously. We used biodegradable spacer (GFLG) for drug attachment for therapy purposes. Comonomer aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (APMA-benzyl-DOTA) was synthesized by reacting N-(3-aminopropylmethacrylamide) (APMA) with p-isothiocyanatobenzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (p-SCN-Bz-DOTA) in dry dimethylsulfoxide (DMSO). The p-SCN-Bz-DOTA was reacted at 1.2 molar excess to APMA. HPMA copolymer conjugates with or without dox were synthesized by a modified two-step procedure. Briefly, in the first step the polymeric precursors containing side chains terminated in DOTA were synthesized by free radical precipitation copolymerization of the monomers of HPMA, APMA-benzyl-DOTA, and MA-GFLG-dox in predetermined molar compositions (Appendix 1, Table 1). All polymerization were carried out in acetone / DMSO using AIBN as the initiator. The ratio of monomers: initiator: solvent in the feed were kept constant at 12.5: 0.6: 86.9 (weight %), respectively. The comonomer mixture was sealed in an ampoule under nitrogen and stirred at 50 °C for 24 h. The polymers were isolated by precipitation of resulting solution into ether. The contents of side chains terminating in DOTA were determined by UV spectrophotometry (λ_{max} = 274 nm). In the second step, the DOTA molecules in the side chain of the polymeric precursors were chelated to gadolinium (Gd) as described elsewhere[3]. Briefly polymer-DOTA conjugates and $GdCl_3.6H_2O$ (1.5:1 molar equivalents relative to the DOTA content) were dissolved in

deionized water. The pH of the solution was maintained at 5-5.5 overnight by gradual addition of 1 N NaOH solution. EDTA disodium salt dihydrate was added into the solutions to chelate the excess Gd. After stirring for 30 min, the milky solution was purified over a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugates. The polymer conjugates were dissolved in deionized water, dialyzed and lyophilized.

The proposed polymers target solid tumors by a passive process of enhanced permeability and retention effect. It is of interest to develop systems that target matastases. Therefore in addition to what was proposed we also synthesized macrophage targetable polymer-linked gadolinium conjugates with implications in imaging and drug delivery to metastatic sites (Appendix 2).

B. Characterization of the copolymers

The characteristics of the copolymers are reported in Table 1 of Appendix 1. Stability of DOTA-Gd was established in literature[4]. All polymer-contrast agent conjugates were characterized for their Gd content by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Galbraith, Knoxville, TN). Doxorubicin content was determined by UV spectrophotometry (λ_{max} = 484 nm). The molecular weight and molecular weight distribution of the polymeric conjugates were estimated by size exclusion chromatography (SEC) on a Superose 12 HR 10/30 column (GE Healthcare, Piscataway, NJ) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare) and HPMA homopolymer fractions of known molecular weight as standards.

The r_1 relaxivity of HPMA copolymer-Gd chelates were calculated from T_1 (relaxation time) measurements at room temperature. Solutions of each sample were diluted in deionized water at four concentrations (from 0.1 to 0.015 mM) and were imaged using 1.5 T MR system (Eclipse, Philips Medical System, Cleveland, OH and Sigma). T_1 was measured using an inversion recovery fast spin echo imaging sequence using inversion times (TI) of 50, 100, 200, 400, 700, 1400, 2000, and 2800 ms, an echo time (TE) of 12 ms, and an echo train length of 8 at a repeat time TR of 6000 ms. All images were obtained from a single axial slice with a 20×15 cm field of view (FOV), 3 mm slice thickness, 256×192 matrix and one excitation. Images were transferred to an independent workstation (SGI, O200) for the calculation of T_1 from the images obtained at various inversion times. T_1 for each solution and deionized water were calculated using MATLAB (The Mathworks, Inc., Natick, MA). The r_1 values of each solution were calculated, using a least squares fit, as the slope of $(1/T_{1,\ solution} - 1/T_{1,\ water})$ versus concentration of contrast agent (mM), where $T_{1,\ solution}$ is the T_1 of each dilution of the contrast agent and $T_{1,\ water}$ is the T_1 of water without contrast agent.

In addition to what was proposed we also characterized the macrophage targetable polymer-linked gadolinium conjugates and evaluated the uptake of these copolymers in vitro (Appendix 2).

C. Concluding year two and strategizing for year 3

In year 3 I plan to continue and finish the proposed research as outlined in the proposal and publish a second manuscript based on our findings.

KEY RESEARCH ACCOMPLISHMENTS

1) Synthesis and characterization of polymer- gadolinium- doxorubicin and polymer-gadolinium conjugates.

2) Comparison of the relaxivity of polymeric contrast agents with and without drug.

3) Synthesis and characterization of macrophage targeted HPMA copolymer - gadolinium conjugates.

4) Comparison of the relaxivity of macrophage targeted polymeric contrast agents with Gd-DOTA.

5) In vitro uptake study of targetable HPMA copolymer gadolinium conjugates.

REPORTABLE OUTCOMES

1) September 06: Successful completion of PhD qualifying examinations.

2) April 2006: Presentation of this research at the University of Maryland Graduate Research Conference in Baltimore (local meeting).

3) June 2006: Presentation of this research at the Annual American Association of Pharmaceutical Scientist Biotechnology Meeting in Boston (National).

4) June 2006: Submission of an original research article to *Molecular Pharmaceutics* (Appendix 2).

From June 5, 2006 to August 13, 2006 I am a summer internship in AstraZeneca Pharmaceuticals in Wilmington, DE. During this period the fellowship research was discontinued. I will continue the work upon return on August 14, 2006.

CONCLUSIONS

In summary progress was made in the synthesis and characterization of polymer-linked contrast agent conjugates with and without doxorubicin (Appendix 1). In addition a series of polymeric

conjugates targetable to macrophages were synthesized and characterized for which a manuscript was submitted for publication (Appendix 2).

REFERENCES

1. Strohalm, J.; Kopecek, J. Poly N-(2-Hydroxypropyl)Methacrylamide. Heterogeneous polymerization. *Angew*. *Makromol. Chem.* **1978**, *70*, 109-118.

2. Ulbrich, K.; Subr, V.; Strohalm, J.; Plocova, D.; Jelinkova, M.; Rihova, B. Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterization. *J. Controlled Rel.* **2000**, 64, 63-79.

3. Wang, D.; Miller, S. C.; Sima, M.; Parker, D.; Buswell, H.; Goodrich, C. H.; Kopeckova, P.; Kopecek, J. The Arthrotropism of macromolecules in adjuvant-induced arthritis rat model: A preliminary study. *Pharm. Res.* **2004**, *21*, 1741-1749.

4. Laurent, S.; Elst, L. V.; Copoix, F.; Muller, R. N. a proton relaxometric protocol for transmetallation assessment. *Invest. Radiol.* **2001**, 36, 115-122.

APPENDIXES

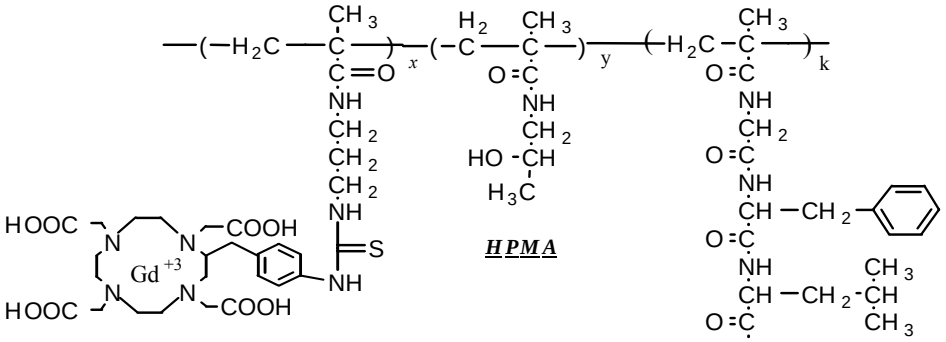
Appendix 1: Chemical structure and characteristics of polymer-linked gadolinium conjugates with and without drug.

Appendix 2: Bahar Zarabi, Anjan Nan, Jiachen Zhuo, Rao Gullapalli, and Hamid Ghandehari, Macrophage targeted N-(2-hydroxypropyl)methacrylamide (HPMA) conjugates for magnetic resonance imaging, *Molecular Pharmaceutics,* Submitted June 2006.

Appendix 1: Chemical structure and characteristics of polymer-linked gadolinium conjugates with and without drug.

Sample	Feed Content (mole%)			Gd content	Dox content	Mw	n[a]	Relaxivity
	HPMA	DOTA	Dox	(mmole/g polymer)		(g/mole)		($s^{-1}mM^{-1}$)
P-Gd	90	10	-	0.41	-	35000	1.6	19.4
P-Gd-Dox	85	10	5	0.19	0.26	34000	1.4	32.5

[a] **Polydispersity**



AMPA-benzyl- DOTA-Gd

HPMA

MA-GFLG-dox

Appendix 2: Bahar Zarabi, Anjan Nan, Jiachen Zhuo, Rao Gullapalli, and Hamid Ghandehari, Macrophage targeted N-(2-hydroxypropyl)methacrylamide (HPMA) conjugates for magnetic resonance imaging, *Molecular Pharmaceutics,* June 2006,Submitted.

Macrophage Targeted *N*-(2-hydroxypropyl)methacrylamide (HPMA) Conjugates for Magnetic Resonance Imaging

Bahar Zarabi[†,‡,*], *Anjan Nan*[†,‡], *Jiachen Zhuo*[đ], *Rao Gullapalli*[‡,đ], *and Hamidreza Ghandehari*[†,‡,**]

[†]Department of Pharmaceutical Sciences, [‡]Center for Nanomedicine and Cellular Delivery, [đ]Department of Radiology, University of Maryland, Baltimore, Baltimore, Maryland 21201.

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TITLE RUNNING HEAD: Macrophage targeted magnetic resonance contrast agent.

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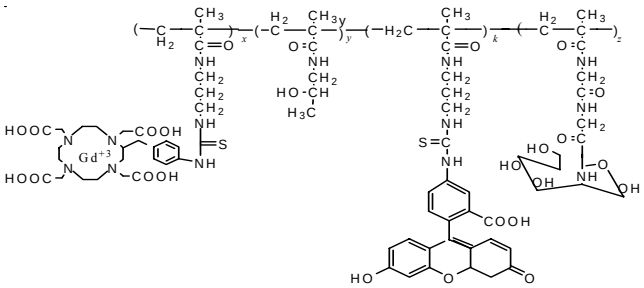

ABSTRACT:

This study describes the synthesis, characterization and *in vitro* evaluation of targetable *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-gadolinium (Gd) chelates for enhanced magnetic resonance imaging (MRI) of macrophages. Copolymers of HPMA, methacryloylglycylglycyl-mannosamine (MA-GG-ManN), aminopropylmethacrylamide-benzyl-1, 4, 7, 10 tetraazacyclododecane-1, 4, 7, 10 tetraacetic acid (APMA-DOTA), and 5-(3(methacryloylaminopropyl)thioureidyl) fluorescein (MA-AP-FITC) were synthesized and characterized. Gd was chelated to the polymeric precursors. The conjugates were characterized for gadolinium content by inductively coupled plasma optical emission spectrometry (ICP-OES) and T_1 relaxivity (r1) at room temperature and 1.5 T. The effect of ManN content on mannose receptor mediated uptake of THP-1 human macrophages was evaluated as a function of time and temperature. The polymer conjugates showed relaxivities in the range of 21.8 to 24.9 $s^{-1}.mM^{-1}Gd$. Relaxivities of the conjugates per mM of Gd were up to 7 times higher than a commercially available MR contrast agent Gd-DOTA. Significantly ($p<0.042$) higher uptake was observed for targeted conjugates compared to non targeted conjugates. The uptake of polymeric conjugates was time and concentration dependent and was mannose receptor-mediated. The increased relaxivity coupled with the ability to target these carriers to cells containing ManN receptors show promise for the application of these agents in clinical MR imaging of macrophage mediated malignancies.

KEYWORDS: HPMA copolymers, Targeted delivery, Contrast agent, Magnetic Resonance Imaging, Relaxivity

Introduction

Activated macrophages play an important role in many pathophysiological processes such as inflammation, autoimmune diseases, cancer, atherosclerosis, neurological disorders, organ rejection, and bacterial soft-tissue infections. Early detection and non invasive monitoring of these conditions are critical for successful intervention. The role of magnetic resonance imaging (MRI) in the detection of macrophage activity is rapidly evolving[1]. Compared to conventional imaging methods such as ultrasound, scintigraphy, computed tomography and radiography, MRI provides a high spatial resolution in detection. Due to this advantage there is an increasing demand for development of sensitive and well tolerated MRI agents that can be rapidly translated from small animal models into patients with diseases that involve mediation of activated macrophages. Findings of several *in vitro* and *in vivo* studies have shown the feasibility and clinical potential of macrophage-specific MR imaging following intravenous administration of iron oxide particles [2-14]. Due to the negative contrast however, differentiation between signal loss caused by iron and native low signal in tissue may be problematic. It is therefore preferable to achieve positive contrast using agents such as Gadolinium (Gd).

Gd-based macromolecular contrast agents provide positive contrast and have several advantages over conventional small molecular weight agents. First, the attachment of multiple contrast agents to a macromolecular carrier such as a polymer increases the local concentration of the agent. Second, due to decrease in molecular motion of the macromolecule, the relaxivity of the contrast agent increases. Third, because of prolonged intravascular retention time of macromolecular contrast agents, imaging of multiple body regions without repeated dosing of

contrast agent is possible[15]. Finally, by passive or active targeting of the macromolecular carrier, it is possible to target the contrast agent to specific cells further enhancing contrast[16].

Mannose receptors are c-type lectin containing multiple carbohydrate-recognition domains[17]. They are expressed primarily on macrophages, dendritic cells as well as some endothelial cells[17, 18]. Due to the enhanced expression of mannose receptors in activated macrophages and the ability of these receptors to recycle, ligand uptake by such cells is essentially continuous allowing accumulation of large quantities of ligands intracellularly[18, 19]. These properties make mannose receptor an attractive target for delivery of diagnostic or therapeutic agents.

N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are nontoxic water-soluble synthetic polymeric carriers that have been extensively evaluated for safety, efficacy and are currently in clinical trials for targeted cancer chemotherapy[20]. Previously HPMA copolymers containing pendant saccharide moieties were evaluated for their bioadhesive properties[21] and for targeted delivery of antileishmanial compounds to liver macrophages[22]. The potential of these copolymers for passive delivery of MR contrast agents has also recently been reported[23, 24]. Active (receptor-mediated) targeting of HPMA-based MR contrast agents to macrophages however remains unexplored. Here we report the synthesis, physicochemical characterization and *in vitro* cellular uptake of HPMA copolymer-Gd chelates containing mannosamine in the side chains for active targeting to macrophages.

Experimental Section

Chemicals and reagents

N, N´-azobisisobutyronitrile (AIBN) and gadolinium (III) chloride hexahydrate ($GdCl_3.6H_2O$) were obtained from Aldrich (Milwaukee, WI, USA). *N*-(3-aminopropylmethacrylamide (APMA) was obtained from Polysciences, Inc. (Warrington, PA, USA). p-isothiocyanatobenzyl-1, 4, 7, 10 tetraazacyclododecane-1, 4, 7, 10 tetraacetic acid (p-SCN-Bz-DOTA) was obtained from Macrocyclics (Dallas, TX, USA). *N, N, N´, N´*- ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA disodium salt dihydrate) was obtained from USB Corporation (Cleveland. OH, USA). Trypan blue stains 0.4% and 2-mercaptoethanol were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was obtained from QBI (Gaithersburg, MD, USA). Phorbol myristate 13-acetate (PMA) was obtained from Promega (Madison, WI, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA) and were of reagent grade.

Cell culture

Human monocyte cell line THP-1 (ATCC TIB 202; ATCC, Manassas, VA) was cultured in modified RPMI 1640 (ATCC) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 0.05 mM 2-mercaptoethanol. Cells were grown at 37 ºC in a humidified atmosphere of 5% CO_2. Phorbol myristate 13-acetate (PMA) 160 nM was applied to monocyte cultures. After incubating with PMA for 48 h, monocytes were differentiated to macrophages. Macrophages were washed with modified RPMI medium containing 10% fetal bovine serum to eliminate the effect of PMA.

Synthesis and characterization of polymer-contrast agent conjugates

Monomer synthesis

N-(2-hydroxypropyl)methacrylamide (HPMA)[25], (5-[3-(methacryloylaminopropyl) thioureidyl] fluorescein) (MA-AP-FITC)[26], and methacryloylglycylglycylmannosamine (MA-GG-ManN)[21] were prepared as described previously. Comonomer aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (APMA-benzyl-DOTA) was synthesized by reacting *N*-(3-aminopropylmethacrylamide) (APMA) with p-isothiocyanatobenzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (p-SCN-Bz-DOTA) in dry dimethylsulfoxide (DMSO). The p-SCN-Bz-DOTA was reacted at 1.2 molar excess to APMA.

Polymer synthesis

HPMA copolymer conjugates with or without ManN were synthesized by a modified two-step procedure. Briefly, in the first step the polymeric precursors containing side chains terminated in DOTA were synthesized by free radical precipitation copolymerization of the monomers of HPMA, APMA-benzyl-DOTA, MA-AP-FITC, and MA-GG-ManN in predetermined molar compositions (Table 1). All polymerization were carried out in acetone / DMSO using AIBN as the initiator. The ratio of monomers: initiator: solvent in the feed were kept constant at 12.5: 0.6: 86.9 (weight %), respectively. The comonomer mixture was sealed in an ampoule under nitrogen and stirred at 50 °C for 24 h. The polymers were isolated by precipitation of resulting solution into ether. The contents of side chains terminating in DOTA were determined by UV spectrophotometry (λ_{max} = 274 nm). In the second step, the DOTA molecules in the side chain of the polymeric precursors were chelated to gadolinium (Gd) as described elsewhere[24]. Briefly polymer-DOTA conjugates and $GdCl_3.6H_2O$ (1.5:1 molar equivalents relative to the DOTA

content) were dissolved in deionized water. The pH of the solution was maintained at 5-5.5 overnight by gradual addition of 1 N NaOH solution. EDTA disodium salt dihydrate was added into the solutions to chelate the excess Gd. After stirring for 30 min, the milky solution was purified over a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugates. The polymer conjugates were dissolved in deionized water, dialyzed and lyophilized. The chemical structure of the macromolecular contrast agent is shown in Figure 1.

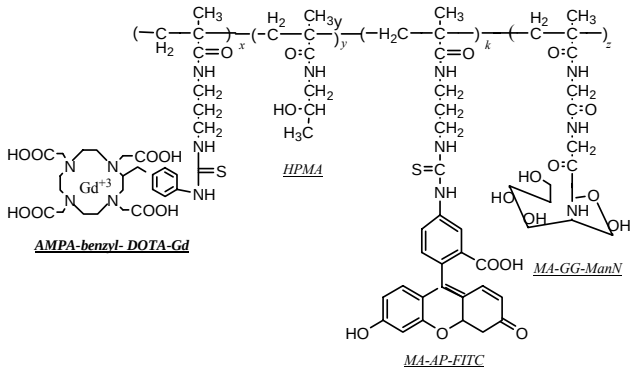

Figure 1. General structure of HPMA copolymer-DOTA (Gd)-ManN-FITC conjugates (HPMA: *N*-(2-hydroxypropyl)methacrylamide; MA-AP-FITC: (5-[3-(methacryloylaminopropyl) thioureidyl] fluorescein); MA-GG-ManN: methacryloylglycylglycylmannosamine; APMA-benzyl-DOTA, aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10-tetraacetic acid; Gd: gadolinium).

Physicochemical characterization

All polymer-contrast agent conjugates were characterized for their Gd content by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Galbraith, Knoxville, TN). The targeting moiety (ManN) content was determined by the Morgan-Elson method and UV spectroscopic measurement as described earlier[27]. Briefly the ManN covalently attached to the

polymer side chains was hydrolyzed under acidic conditions followed by complexation of the hydrolyzed sugar with *p*-dimethylaminobenzaldehyde to yield a colored complex, which was determined spectrophotometrically at 585 nm. DOTA and FITC content of the final conjugates were determined by UV spectrophotometry at 274 nm and 492 nm respectively. The molecular weight and molecular weight distribution of the polymeric conjugates were estimated by size exclusion chromatography (SEC) on a Superose 12 HR 10/30 column (GE Healthcare, Piscataway, NJ) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare) and HPMA homopolymer fractions of known molecular weight as standards.

Relaxivity measurements

The r_1 relaxivity of HPMA copolymer-Gd chelates were calculated from T_1 (relaxation time) measurements at room temperature. Solutions of each sample were diluted in deionized water at four concentrations (from 0.1 to 0.015 mM) and were imaged using 1.5 T MRsystem (Eclipse, Philips Medical System, Cleveland, OH and Sigma). T_1 was measured using an inversion recovery fast spin echo imaging sequence using inversion times (TI) of 50, 100, 200, 400, 700, 1400, 2000, and 2800 ms, an echo time (TE) of 12 ms, and an echo train length of 8 at a repeat time TR of 6000 ms. All images were obtained from a single axial slice with a 20×15 cm field of view (FOV), 3 mm slice thickness, 256×192 matrix and one excitation. Images were transferred to an independent workstation (SGI, O200) for the calculation of T_1 from the images obtained at various inversion times. T_1 for each solution and deionized water were calculated using MATLAB (The Mathworks, Inc., Natick, MA). The r_1 values of each solution were calculated, using a least squares fit, as the slope of $(1/T_{1,\ solution} - 1/T_{1,\ water})$ versus concentration of contrast

agent (mM), where $T_{1,\ solution}$ is the T_1 of each dilution of the contrast agent and $T_{1,\ water}$ is the T_1 of water without contrast agent.

In vitro evaluation of polymer-contrast agent conjugates

Macrophage uptake studies

FITC (fluorescein-5-isothiocyanate) was used as a fluorescent probe to measure biorecognition and uptake of polymeric conjugates in a model THP-1 human monocytes. Before analysis cells were seeded on 96-well culture plates at a concentration of 1×10^5 cells/ well and treated with 160 nM PMA in modified RPMI 1640 (10% fetal bovine serum) for 48 h at 37 ºC and in humidified atmosphere of 5% CO_2. Upon treatment with PMA, THP-1 cells adhered to the dish, as the first indication of differentiation to macrophages[28, 29]. Before the uptake study, cells were washed with modified RPMI 1640 (10% fetal bovine serum) to stop the effect of PMA. 100 µl of HPMA copolymer-DOTA conjugates (with and without Gd) in modified RPMI 1640 (10% FBS) were added to obtain final concentrations of 2, 4, and 8 µM (ManN equivalent), respectively, for each sample. Experiments were performed at 3, 6, and 24 h to determine time dependent effects on uptake. The same experiment was performed with 1.15 and 0.58 µM (FITC equivalent), respectively, for each sample to compare the % of uptake with control. At each time point the overlay was removed and cells washed 2 times with PBS. 100 µl of modified RPMI 1640 (without FBS) was subsequently added to each well and the total fluorescence associated with the cells was determined directly on a SPECTRAmax Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) (Ex/Em 492/520). Experiments were performed at 37 ºC

and 4 ºC to determine temperature dependent active receptor mediated uptake. Polymers with and without Gd were compared to determine its effect on uptake.

To confirm active mannose receptor mediated uptake, additional experiments were performed with macrophages preincubated with 100 mM[19] of ManN solution for 3 h. The uptake of the conjugates in all experiments was expressed as % of fluorescence in the feed after correcting for background. Statistical significance of differences in uptake between different samples was analyzed using student t-test.

Quenching of extracellular fluorescence

The concentration of trypan blue required to completely quench extracellular fluorescence was first determined by exposing of 100 µl/well of Sample P_1 (Table 1) (2, 4, 8 µM equivalent of FITC) to 100 µl of serial dilution of the dye (62.5-4000 µg/ml) in 96 well plate. The fluorescence intensity was measured directly in the wells using fluorescent plate reader (Ex/Em 492/520). Wells containing only Sample P_1 (Table 1) (2, 4, 8 µM equivalent of FITC) were used as controls to indicate complete quenching.

In subsequent experiments after incubation of macrophages with the polymer conjugates for 3 h, extracellular fluorescence was quenched by adding 100 µl of trypan blue (4000 µg/ml). The dye was removed after 1 min and cells were washed two times with PBS. 100 µl of modified RPMI 1640 (without fetal bovine) was subsequently added to each well and the intensity of intracellular fluorescence was measured directly in the wells.

Results

Synthesis and characterization of HPMA copolymer- contrast agent conjugates

A series of HPMA-DOTA-(Gd)-FITC conjugates were synthesized with incremental variation in targeting moiety (ManN) content (Figure 1, Table 1). As control a conjugate without targeting moiety (Sample P_0, Table 1) was synthesized. The incorporations of the chelating (APMA-DOTA), reporter (MA-AP- FITC) and targeting (MA-GG-ManN) comonomers were between 71-92%, 66-96% and 78-98% respectively of the feed comonomer content. Subsequent chelation of the DOTA side chains of the conjugates with Gd resulted in Gd incorporation efficiency of 52-75% of the DOTA molecules per polymer backbone. All HPMA-linked Gd conjugates exhibited relaxation (r_1) values up to seven times greater than a commercially available Gd-DOTA contrast agent (Dotarem®)[30] (Table 1). The estimated weight average molecular weight of the polymers was between 52-63 kDa with poly dispersity index ranging from 1.6-1.8 (Table 1) which was typical of similar polymeric conjugates reported in the literature[22].

Table 1. Physicochemical characteristics of targetable HPMA copolymer - contrast agent conjugates.

Sample[a]	Feed comonomer composition (mole%)				Polymer Characteristics (mmole/g polymer)				Mw[b] (g/mole)	n[c]	Relaxivity (s^{-1}. mM^{-1} Gd)
	HPMA	ManN	DOTA	FITC	DOTA content[b]	ManN content[b]	FITC content[b]	Gd content[d]			
P_0	88	0	10	2	0.452±0.01	0	0.053±0.02	0.43	52000	1.6	21.8
P_1	86	2	10	2	0.332±0.01	0.097±0.03	0.083±0.02	0.25	63000	1.6	21.5
P_2	84	4	10	2	0.325±0.02	0.199±0.04	0.095±0.02	0.21	59000	1.8	24.4
P_3	80	8	10	2	0.313±0.01	0.272±0.05	0.083±0.02	0.21	58000	1.7	24.4
P_4	72	16	10	2	0.290±0.02	0.498±0.04	0.072±0.02	0.15	58000	1.8	24.9
Gd-DOTA	-	-	-	-	-	-	-	-	-	-	3.4

[a] For structures of polymer-contrast agent conjugates see Figure 1.

[b] Weight average molecular weight of polymer precursor.

[c] Polydispersity index.

[d] Polymer contrast agent conjugate.

Macrophage uptake studies

Time and concentration dependent uptake

The time and concentration dependent uptake of polymeric conjugates by macrophages was evaluated (Figure 2). The fluorescence values (expressed as % of feed content) corresponding to the uptake of conjugates were compared at three different concentrations of 2, 4 and 8 μM (equivalent of ManN) (Figure 2, Panels a, b, c). The uptake of all the targetable conjugates increased with increase in ManN concentration at all the time points. After 24 h the uptake of 4 mole% or higher ManN containing polymers was significantly ($p<0.040$) higher than after 3 and 6 h. This was observed at both 4 and 8 μM concentrations of ManN. However at 2 μM concentration, the uptake after 24 h was only significantly different ($p< 0.024$) from uptake after 3 and 6 h for Sample P_4 (Table 1). There was no significant difference in uptake between 3 and 6 h at any concentration.

Effect of targeting moiety

Conjugates with 4 mole % or higher of ManN (P_2 -P_4) resulted in significantly ($p<0.017$) higher uptake than 2 mole% ManN containing conjugate (P_1) at the same equivalent concentrations of targeting

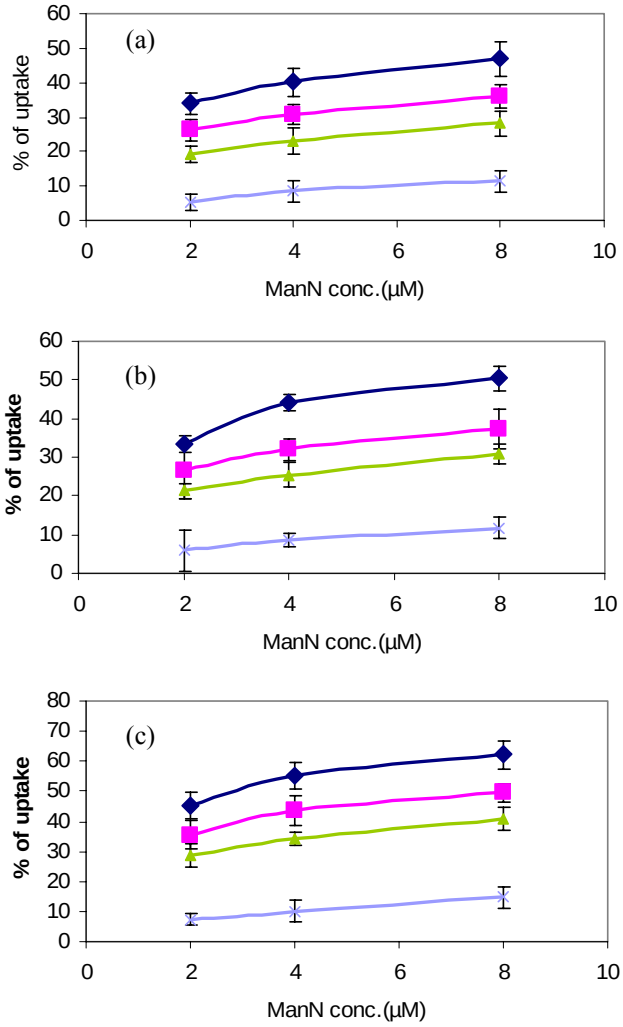

Figure 2. Time dependent uptake of targetable HPMA copolymer - contrast agent conjugates at 37ºC after: a)3h; b)6h; and c)24h. P_4 (♦); P_3 (■); P_2 (▲); P_1 (×).Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

moiety and at all time points studied, namely 3, 6 and 24 h. At 8 and 4 μM (equivalent of ManN) concentrations, 16 mole% ManN containing conjugate showed significantly higher uptake ($p<0.041$) than 8 mole% after 6 and 24 h. The uptake of polymeric conjugates with 4 mole% or

higher ManN content was significantly ($p<0.042$) higher than control nontargeted conjugate (P_0) at the same equivalent concentration of FITC after 3 h incubation (Figure 3). Polymer with 2 mole% ManN did not show significant uptake compared to the control without targeting moiety (Figure 3). Incubation of macrophages with polymeric conjugates at 4ºC (Figure 4) resulted in significantly reduced uptake when compared to those carried out at 37°C suggesting the involvement of an active receptor mediated process.

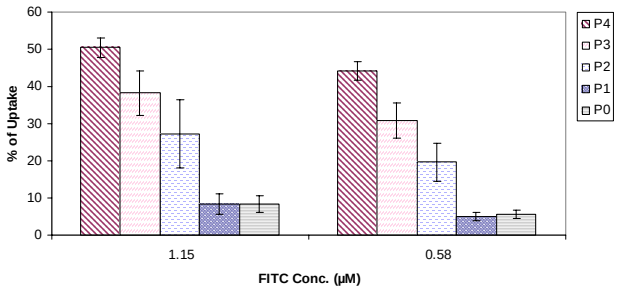

Figure 3. % of uptake of HPMA copolymer - contrast agent conjugates at the same equivalent concentration of FITC. Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

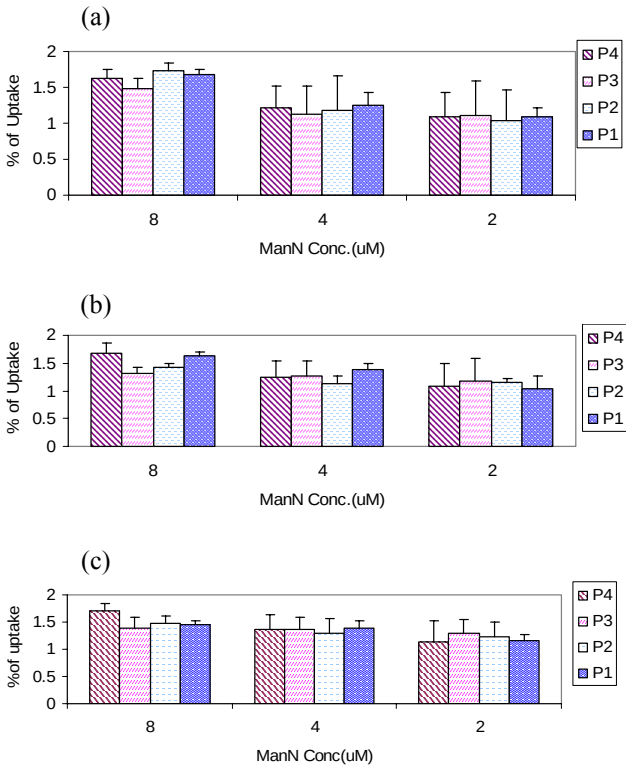

Figure 4. Time dependent uptake of targetable HPMA copolymer - contrast agent conjugates at 4 ºC after: a)3h; b)6h; and c)24h. Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

Effect of Gd on uptake

Uptake of polymeric conjugates with and without Gd was compared to each other after 3h (Figure 5). Polymer-chelated Gd showed higher trend of uptake but the difference between conjugate uptake with and without Gd was not significant.

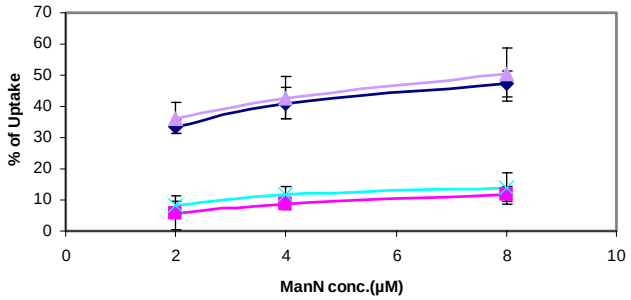

Figure 5. Effect of Gd on uptake of HPMA copolymer - contrast agent conjugates. P_4 (♦); P_1 (■); P_4-Gd (▲); P_1-Gd (×). Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of samples see Figure 1 and Table 1.

Evidence of mannose receptor mediated uptake

The uptake of polymeric conjugates with 4, 8 and 16 mole% of targeting moiety at 2, 4, 8 μM after 3 h was inhibited by 65-85% upon pre-incubation with free ManN (Figure 5). However, the uptake of P_1 before and after treatment did not change significantly. Figure 6 showed that after mannose treatment the uptake of P_4, P_3, and P_2 was the same as P_1. These results suggest that uptake of polymeric conjugates is mediated primarily by mannose receptors. The lack of

complete inhibition indicates that a secondary mechanism such as adsorptive endocytosis of the polymers may also exist.

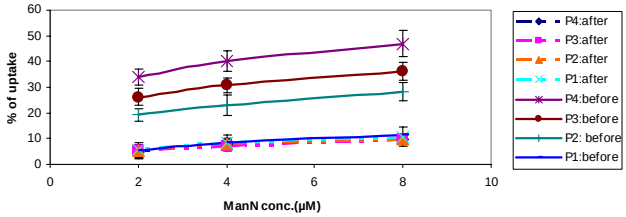

Figure 6. Effect of pre-incubation with free ManN on the uptake of HPMA copolymer - contrast agent conjugates by macrophages. Dashed lines show % of uptake of polymers in the presence of ManN. Full lines show % of uptake of polymers in the absence of ManN. Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

Extracellular fluorescence quenching

In this study, we used the trypan blue dye technique to quench the extracellular fluorescence [31, 32]. Complete quenching of FITC fluorescence was obtained by 4000 μg/ml of trypan blue (data has not been shown). This concentration was subsequently used in uptake measurements. Uptake studies carried out with quenching of extracellular fluorescence using trypan blue resulted in the decrease of measured uptake values by 12.5-17.9% for all the polymers (Figure 7). The decrease in % of uptake after incubating the cells with trypan blue showed the non-specific adhering of polymeric conjugates to the surface of macrophages.

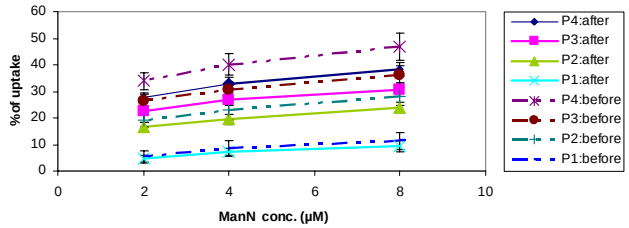

Figure 7. Effect of extracellular fluorescence quenching on the uptake of HPMA copolymer - contrast agent conjugates by macrophages. Dashed lines show % of uptake of polymers before quenching. Full lines show % of uptake of polymers after quenching. Uptake is expressed as mean of three samples ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

Discussion

Macrophages are a major component of the mononuclear phagocyte system that play a critical role in the initiation, maintenance, and resolution of inflammation. Activated macrophages secrete multiple potent mediators of inflammation and tissue destruction, including proinflammatory cytokines (e.g. IL-1, IL-6, TNF-a), chemokines, prostaglandins, metalloproteinases, and reactive oxygen species[33, 34]. Further, activated macrophages are known to participate in antigen presentation, and thereby they are thought to contribute to the activation and proliferation of antigen specific T-cells and their consequent destructive activities[33, 35, 36]. Because macrophages produce a wide range of biologically active molecules participated in both beneficial and detrimental outcomes in inflammation, therapeutic interventions targeting macrophages and their products may open new avenues for controlling inflammatory diseases. However understanding the underlying mechanisms of macrophage action is still in question. Current strategies mostly involve invasive procedures such as blood sampling, biopsy, etc. Non invasive external imaging techniques such as MRI offer methods for the measurement of cell behavior and biochemical events in situ and can be valuable tools for early detection and diagnosis of the diseases where macrophages are involved. The use of MR contrast agents such as Gd is limited by the sheer amount required to obtain a good signal for detection. An approach

that would selectively localize a high concentration of contrast agents in the activated macrophages without compromising their essential functions can enhance contrast signal to background ratio significantly. In addition such an approach could also be considered as a surrogate for a similar delivery of a therapeutic payload, e.g., anti-cancer, anti inflammatory or antiarthritic drugs or tumor vaccine to induce an immune response[35]. The macrophage mannose receptor, exclusively expressed on activated macrophages, can be used to target and localize large amounts of contrast agents for diagnostic purposes.

In this study, we evaluated macrophage targetable macromolecular contrast agents consisting of gadolinium (Gd) chelated to the backbone of water-soluble HPMA copolymers. The HPMA copolymer backbone contains a multivalency of mannosamine molecules as targeting ligands specific to the macrophage mannose receptors. The hypothesis of the study was that by active targeting of Gd-polymer conjugates to the macrophages it is possible to significantly increase accumulation of contrast agent resulting in a higher macrophage to background ratio of accumulation. HPMA copolymers are advantageous as macromolecular carriers because of the ability to tailor make the polymer backbone and control the content of side chains by facile chemical manipulations. As a first step towards development of HPMA copolymer - ManN conjugates for targeted delivery of Gd to macrophages, we synthesized and characterized a series of these conjugates with incremental variation in their targeting moiety content. The purpose was to evaluate the effect of a range of targeting moiety content on the extent of biorecognition and uptake by macrophages. This would help in identification of a lead conjugate with optimum ManN content for highest macrophage specific targeting.

Our results demonstrated successful synthesis and characterization of HPMA based macrophage targetable macromolecular contrast agents. The molecular size of the conjugates ranged between 50-60 kDa which is large enough to be retained in the macrophages once internalized[37]. Observed relaxivities for HPMA copolymer contrast agent conjugates (Table 1) were improved over commercially available contrast agents Gd-DOTA. Conjugation of Gd-DOTA to larger macromolecules is known to increase relaxivity by reducing rotational correlation time[38]. This has been observed for many Gd-DOTA complexes[39, 40, 41] and is similarly observed for HPMA based contrast agents. Importantly the advantage of HPMA conjugates over Gd-DOTA will be the larger molecular size which may result in longer retention time in the macrophages. Consequently it may be possible to obtain enhanced long term imaging data due to sustained incremental accumulation of the macromolecular agent compared to Gd-DOTA.

THP-1 cells are well known for their phagocytic properties[42] and expression of mannose receptors[43]. As a result this cell line was chosen as a model for our studies. A fluorimetric phagocytosis assay was adopted using FITC fluorescence to compare the uptake of the various mannose containing polymeric conjugates.

The uptake data of ManN containing HPMA copolymer-DOTA conjugates suggest the involvement of mannose receptors in recognition of the conjugates by macrophages. With an increase in the ManN content of the conjugates there was a significant ($p<0.042$) increase in their uptake which suggests that the uptake mechanism may be an active receptor mediated process in the recognition and internalization of these polymeric conjugates. The same uptake studies carried out at 4°C showed little or no uptake further confirming the involvement of an active process (Figure 4). A 4 mole% or higher ManN content of the conjugates resulted in significantly

higher uptake compared to nontargeted conjugates, further suggesting active uptake. It is well established that the affinity of receptor ligand binding in active targeting is often enhanced by the multivalency of the ligands [44]. A significant increase in uptake from 2 to 4 mole% ManN polymer is indicative of this multivalent effect. The uptake of 2 mole% ManN conjugate was comparable to the conjugate without any targeting moiety (Figure 3). These results suggest that the number of targeting moiety per polymeric backbone can influence the uptake through ManN receptors. Further mechanistic studies need to be done to evaluate the role and quantify the ManN concentration required for maximum uptake.

An active process such as receptor mediated binding and internalization typically demonstrates saturabiltiy. In the presence of free ManN the uptake of all targetable polymeric conjugates decreased by 65-85% (Figure 6). These results suggest the competitive inhibition effect by ManN and therefore strongly support the conclusion that uptake of polymeric conjugates is mediated primarily by mannose receptors.

The limited uptake by the non targetable HPMA conjugate suggests the possible involvement of a passive endocytosis mechanism as well. The involvement of passive endocytosis for polymeric conjugates is further suggested by time dependent studies (Figure 2). The uptake after 24 h for 4 mole% or higher ManN containing polymers was significantly ($p<0.040$) higher than after 3 and 6 h at 8 and 4 μM (equivalent of ManN). No significant difference between 3 and 6 h at any concentrations was observed possibly since the passive uptake of macromolecules is usually a slower kinetic process. These observations are in agreement with previous results on similar polymeric carriers for the delivery of antileishmanial agents[22].

The current studies demonstrate the potential of HPMA copolymer-ManN-Gd conjugates as macromolecular contrast agents for enhanced MR imaging in conditions where activated macrophages are involved. The linear flexible and hydrated chains of HPMA copolymers can provide higher molar relaxivities. Covalent attachment of targeting moieties with control over content can allow optimization of macrophage localization. Control over molecular weight and charge can allow control over pharmacokinetics and biodistribution[45, 46]. Finally such conjugates can be used for simultaneous delivery of drugs and imaging agents to allow optimization of therapy to target sites.

Conclusions

HPMA copolymer-Gd conjugates containing ManN were synthesized and characterized. *In vitro* studies demonstrated active mannose receptor mediated uptake of the conjugates by macrophages as well as by passive endocytosis. The multivalency of ManN units on the polymer backbone resulted in significantly higher uptake than non targetable conjugates. The conjugates showed relaxivity values ranging from 6.3 to 7.3 fold higher than Gd. These results demonstrate the potential of macrophage-targeted HPMA copolymers for delivery of MR contrast agents.

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REFERENCES:

1. Weinmann, H. J.; Ebert, W.; Misselwitz, B.; Schmitt-Willich, H. Tissue-Specific MR Contrast Agents. *Eur. J. Radiol*. **2003**, *46*, 33-44.
2. Weissleder, R.; Elizondo, G.; Wittenberg, J.; Lee, AS.; Josephson, L.; Brady, TJ. Ultrasmall Superparamagnetic Iron Oxide: An Intravenous Contrast Agent For Assessing Lymph Nodes With MR Imaging. *Radiology.* **1990,** *175*, 494-498.
3. Weissleder, R.; Elizondo, G.; Wittenberg, J.; Rabito, C. A.; Bengele, H. H.; Josephson, L.; Ultrasmall Superparamagnetic Iron Oxide: Characterization Of A New Class Of Contrast Agents For MR Imaging. *Radiology*. **1990**, *175*, 489-493.
4. Weissleder, R.; Cheng, H. C.; Bogdanova, A.; Bogdanov, A.; Jr. Magnetically Labeled Cells Can Be Detected By MR Imaging. *J. Magn. Reson. Imaging*. **1997**, *7*, 258-263.
5. Schulze, E.; Ferrucci, J. T.; Jr, Poss, K.; Lapointe, L.; Bogdanova, A.; Weissleder, R. Cellular Uptake And Trafficking Of A Prototypical Magnetic Iron Oxide Label In Vitro. *Invest. Radiol*. **1995**, *30*, 604-610.
6. Dousset, V.; Delalande, C.; Ballarino, L.; Et Al. In Vivo Macrophage Activity Imaging In The Central Nervous System Detected By Magnetic Resonance. *Magn. Reson. Med.* **1999**, *41*, 329-333.
7. Hauger, O.; Delalande, C.; Deminiere, C.; Et Al. Nephrotoxic Nephritis And Obstructive Nephropathy: Evaluation With MR Imaging Enhanced With Ultrasmall Superparamagnetic Iron Oxide—Preliminary Findings In A Rat Model. *Radiology.* **2000**, *217*, 819-826.
8. Kanno, S.; Wu, Y. J.; Lee, P. C.; Et Al. Macrophage Accumulation Associated With Rat Cardiac Allograft Rejection Detected By Magnetic Resonance Imaging With Ultrasmall Superparamagnetic Iron Oxide Particles. *Circulation.* **2001**, *104*, 934-938.
9. Ruehm, S. G.; Corot, C.; Vogt, P.; Kolb, S.; Debatin, J. F. Magnetic Resonance Imaging Of Atherosclerotic Plaque With Ultra small Superparamagnetic Particles Of Iron Oxide In Hyperlipidemic Rabbits. *Circulation.* **2001**, *103*, 415-422.
10. Rausch, M.; Baumann, D.; Neubacher, U.; Rudin, M. In-Vivo Visualization Of Phagocytotic Cells In Rat Brains After Transient Ischemia By USPIO. *NMR. Biomed.* **2002**; *15*, 278-283.

11. Stets, C.; Brandt, S.; Wallis, F.; Buchmann, J.; Gilbert, F. J.; Heywang-Kobrunner, S.H. Axillary Lymph Node Metastases: A Statistical Analysis Of Various Parameters In MRI With USPIO. *J. Magn. Reson. Imaging.* **2002**, *16*, 60-68.

12. Sigal, R.; Vogl, T.; Casselman, J.; Et Al. Lymph Node Metastases From Head And Neck Squamous Cell Carcinoma: MR Imaging With Ultrasmall Superparamagnetic Iron Oxide Particles (Sinerem MR)-Results Of A Phase-III Multicenter Clinical Trial. *Eur. Radiol.* **2002**, *12*, 1104-1113.

13. Daldrup-Link, H.E.; Rummeny, E.J.; Ihssen, B.; Kienast, J.; Link, T. M. Iron-Oxide-Enhanced MR Imaging Of Bone Marrow In Patients With Non-Hodgkin's Lymphoma: Differentiation Between Tumor Infiltration And Hypercellular Bone Marrow. *Eur. Radiol.* **2002**, *12*, 1557-1566.

14. Kaim, A. H.; Wischer, T.; O'Reilly, T.; Et Al. MR Imaging With Ultra small Superparamagnetic Iron Oxide Particles In Experimental Soft-Tissue Infections In Rats. *Radiology.* **2002**, *225*, 808-814.

15. Bogdanov, Jr. A.A.; Weissleder, R.; Frank, H. W.; Bogdanova, A. V.; Nossif, N.; Schaffer, B. K.; Tsai, E. A New Macromolecule As A Contrast Agent For MR Angiography: Presentation, Properties, And Animal Studies. *Radiology*. **1993**, *187*, 701-706.

16. Orang-Khadivi, K.; Piereceet, B.L.; Ollom, C. M.; Floyd, L. J.; Siegel, R. L.; Williams, R. F. New Magnetic Resonance Imaging For The Detection Of Breast Cancer. *Breast Cancer Research And Treatment.* **1994,** *32*, 119-135.

17. Taylor, M. E.; Evaluation Of A Family Of Receptors Containing Multiple C-Type Carbohydrate-Recognition Domains. *Glycobiology.* **1997**, *7*, v-viii.

18. Stahl, P. D.; Ezekowitz, R.A.B. The Mannose Receptor Is A Pattern Recognition Receptor Involved In Host Defense. *Curr. Opin. Immunol*. **1998**, *10*, 50-55.

19. Sallusto, F.; Cella, M.; Danieli, C.; Lanzavecchia, A.; Denderic Cells Use Macropinocytosis And The Mannose Receptor To Concentrate Macromolecules In The Major Histocompatibility Complex Class II Compartment: Down Regulation By Cytokines And Bacteria Products. *J. Exp.Med*. **1995**, *182*, 389- 400.

20. Kopecek, J.; Kopeckova, P.; Minko, T.; Lu, Z. HPMA Copolymer-Anticancer Drug Conjugates: Design, Activity, And Mechanism Of Action. *Eur. J. Pharm. Biopharm*. **2000**, *50*, 61-81.

21. Rathi, R. C.; Kopeckova, P.; Rihova, B.; Kopecek, J. N-(2-Hydroxypropyl)Methacrylamide Copolymers Containing Pendant Saccharide Moieties: Synthesis And Bioadhesive Properties, *J. Polym. Sci., A, Polym. Chem.* **1991**, *29*, 1895–1902.

22. Nan, A.; Croft, S.L.,Yardley, V.; Ghandehari, H.; Targetable Water-Soluble Polymer-Drug Conjugates For The Treatment Of Visceral Leishmaniasis. *J. Control. Release*. **2003**, *94*, 115-127.

23. Huang, Y.; Nan, A.; Rosen, G. M.; Winalski, C. A.; Schneider, E.; Tsai, P.; Ghandehari, H. N- (2-Hydroxypropyl)Methacrylamide (HPMA) Copolymer-Linked Nitroxide: Potential Magnetic Resonance Contrast Agents. *Macromol. Biosci.* **2003**, *3*, 647-652.

24. Wang, D.; Miller, S. C.; Sima, M.; Parker, D.; Buswell, H.; Goodrich, C. H.; Kopeckova, P.; Kopecek, J. The Arthrotropism Of Macromolecules In Adjuvant-Induced Arthritis Rat Model: A Preliminary Study. *Pharm. Research.* **2004**, *21*, 1741-1749.

25. Strohalm, J.; Kopecek, J. Poly N-(2-Hydroxypropyl)Methacrylamide. 4. Heterogeneous Polymerization. *Angew. Makromol. Chem.* **1978**, *70*, 109-118.

26. Omelyanenko, V.; Kopeckova, P.; Gentry, C.; Kopecek, J. Targetable HPMA Copolymer-Adriamycin Conjugates. Recognition, Internalization, And Subcellular Fate. *J. Control. Release*. **1998**, *53*, 25-37.

27. Van De Loo, H. M. An Improved Method For The Quantitative Determination Of Hexosamines According To Elson And Morgan, *Anal. Biochem*. **1976**, *76*, 556-60.

28. Perieto, J.; Eklund, A.; Patarroyo, M.; Regulated Expression Of Integrins And Other Adhesion Molecules During Differentiation Of Monocytes Into Macrophages. *Cell Immun.* **1994**, *156*, 191-211.

29. Schwende, H., Fitzke, E., Ambs, P., Dieter, P., Differences In The State Of Differentiation Of THP-1 Cells Induced By Phorbol Ester And 1, 25-Dihydroxyvitamin D3. *J. Leukoc. Biol.* **1996**, *59*, 555-561.

30. GE Healthcare Website: http://www.amershamhealth.com (Visited June 2006)

31. Hed, J.; Hallden, G.; Johansson, S. G. O.; Larsson, P.; The Use Of Fluorescence Quenching In Flow Cytofluorometry To Measure The Attachment And Ingestion Phases In Phagocytosis In Peripheral Blood Without Prior Cell Separation. *J. Immunol. Methods.* **1987**, *101*, 119-125.

32. Sahlin, S.; Hed, J.; Runquist, I.; Differentiation Between Attached And Ingested Immune Complexs By A Fluorescence Quenching Cytofluorometric Assay. *J. Immunol. Methods.* **1983**, *60*, 115-124.

33. Kinne, R.W.; Brauer, R.; Stuhlmuller, B.; Palombo-Kinne, E.; Burmester, G. R.; Et Al. Macrophages In Rheumatoid Arthritis, *Arthritis Res.* **2000**, *2*, 189–202.

34. Kmiec, Z. Cooperation Of Liver Cells In Health And Disease. *Adv. Ana.T Embryol*. Cell Biol.**2001**, *161*, iii–xiii, 1–151.

35. Bresnihan, B. Pathogenesis Of Joint Damage In Rheumatoid Arthritis. *J. Rheumatol.* **1999**, *26*, 717-719.

36. Burmester, G. R.; Stuhlmuller, B.; Keyszer,G.; Kinne, R. W. Mononuclear Phagocytes And Rheumatoid Synovitis. Master-Mind Or Workhorse In Arthritis? *Arthritis Rheum.* **1997**, *40*, 5–18.

37. Kobayashi, H.; Kawamoto, S.; Bernardo, M.; Brechbiel, M. W.; Knopp, M. V.; Choyke, P. L. Delivery Of Gadolinium-Labeled Nanoparticles To The Sentinel Lymph Node: Comparison Of The Sentinel Node Visualization And Estimations Of Intra-Nodal Gadolinium Concentration By The Magnetic Resonance Imaging. *J. Control. Release.* **2006**, *111*, 343-351.

38. Meyer, D.; Schaefer, M.; Bonnemain, B. Gd-DOTA, A Potential MRI Contrast Agent. Current Status Of Physicochemical Knowledge. *Invest. Radiol.* **1988**, *23* (Suppl. 1), S232-235.

39. Caravan, P.; Greenfield, M.; Li, Z.; Sherry, A. The Gd3+ Complex Of A Fatty Acid Analogue Of DOTP Binds To Multiple Albumin Sites With Variable Water Relaxivities. *Inorg. Chem.* **2001**, *40*, 6580-6587.

40. Caravan, P.; Ellison, J.; Mcmurry, T.; Lauffer, R. Gadolinium (III) Chelates As MRI Contrast Agents: Structure, Dynamics, And Applications. *Chem. Rev.* **1999**, *99*, 2293-2352.

41. Nivorozhkin, A.; Kolodziej, A.; Caravan, P.; Greenfield, M.; Lauffer, R.; Mcmurry, T. Enzyme-Activated Gd3+ Magnetic Resonance Imaging Contrast Agents With A Prominent Receptor-Induced Magnetization Enhancement. *Angew. Chem., Int.Ed.* **2001**, *40*, 2903-2906.

42. Tscuchiya, S.; Establishment And Characterization Of A Human Acute Monocytic Leukemia Cell Line (THP-1). *Int. J. Cancer.* **1980**, *26*, 171-176.

43. Diaz-Sivestre, H.; Espinosa-Cueto, P.; Sanchez-Gonzalez, A.; Esparza-Ceron, M. A.; Pereira-Suarez, A. L.; Bernal-Fernandez, G.; Espitia, C.; Mancilla, R. The 19-Kda Antigen Of Mycobacterium Tuberculosis Is A

Major Adhesion That Binds The Mannose Receptor Of THP-1 Monocytic Cells And Promotes Phagocytosis Of Mycobacteria. *Microb. Pathog.* **2005**, *39*, 97-107.

44. Roseman, D. S.; Baenziger, J. U. The Mannose/N-Acetylgalactosamine-4-SO4 Receptor Displays Greater Specificity For Multivalent Than Monovalent Ligands. *J. Biol. Chem.* 2001, *276*, 17052-17057.

45. Mitra, A.; Nan, A.; Ghandehari, H.; Mcneill, E.; Mulholland, J.; Line, B. R.Technetium-99m-Labeled N-(2-Hydroxypropyl)methacrylamide Copolymers: Synthesis, Characterization And In Vivo Biodistribution. *Pharm. Res.* **2004**, 21, 1153-1159.

46. Kissel, M.; Peschke, P.; Subr, V.; Ulbrich, K.; Schuhmacher, J.; Debus, J.; Friedrich, E. Synthetic Macromolecular Drug Carriers: Biodistribution Of Poly [(N-2-Hydroxypropyl)Methacrylamide] Copolymers And Their Accumulation In Solid Rat Tumors. *PDA J. Pharm. Sci. & Tech.* **2001**, *55*,191–201.